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(54) Title: DELIVERY OF TOLEROGENIC ANTIGENS VIA EDIBLE PLANTS OR PLANT-DERIVED PRODUCTS (57) Abstract Autoantigens and allergens can be expressed in transgenic plants, and the plants, or products derived therefrom, used as foods or beverages to prevent or treat autoimmune diseases or allergic reactions.		

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**DELIVERY OF TOLEROGENIC ANTIGENS VIA EDIBLE PLANTS
OR PLANT-DERIVED PRODUCTS**

This application claims the benefit under §119(e) of prior U.S. provisional application 60/023,973, filed August 15, 1996, which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for producing autoantigens and allergens in plants and the use of the plants or plant-derived products as medicinal food in treatment or prophylaxis of autoimmune diseases and allergic reactions.

Description of the Background Art

The cellular immune response has evolved to distinguish normal cells (self) from aberrant tissues such as virus-infected cells (non-self). It is believed that macrophages process viral or foreign antigens and present them to a population of T lymphocytes called helper cells (T_H), which characteristically express the marker molecule CD4. Two types of CD4 cells have been defined in mice (19). T_{H1} cells mediate delayed-type hypersensitivity and are thought to assist T cells, which bear the molecular marker molecule, CD8, in becoming cytotoxic T lymphocytes (CTL, T_C), while T_{H2} cells collaborate with B lymphocytes in the production of antibodies. T_{H1} and T_{H2} cells can be distinguished from one another by the type of cytokine they produce. Specifically, T_{H1} cells secrete proinflammatory cytokines, such as interleukin-2 (IL2), γ -interferon (IFN- γ) and tumor necrosis factor (TNF) while T_{H2} cells produce anti-inflammatory cytokines, such as IL-4, IL-6 and IL-10. Cytokines secreted from T helper cells mediate cellular interactions interacting with receptors on appropriate cells, e.g., CTLs expressing IL-2 receptors enable them to respond to T_{H1} cells. The ratio of T_{H1} to T_{H2} cells generated during an immune response varies according to the pathogen.

Lymphocytes require two signals to become activated. One is antigen-specific and involves the recognition, by receptors expressed on T cells (TCRs), of degraded foreign antigen in association with class I or class II molecules, encoded in the

MHC (20). The second signal is not antigen-specific and is mediated by soluble cytokines. Most pertinent to this invention is the observation that lymphocytes receiving stimulation from specific antigens in the absence of the second signal, not only fail to become activated but are refractory to further stimuli (21).

The ability of T cells to specifically recognize foreign antigens while simultaneously being unresponsive or tolerant of self antigens is not totally understood, but appears to be determined by two selection processes which occur in the thymus. During T cell development, when these cells undergo gene rearrangements to produce TCRs, only T cells which can interact with self MHC molecules and foreign peptides are positively selected, and subsequently, in a second round of negative selection, those TCRs which interact very strongly with self antigens expressed in the thymus are eliminated or deleted, thus ensuring self tolerance. T cells are not, however, exposed to all self-antigens in the thymus, since many proteins have specialized functions and are synthesized only in specific tissues. Naive T cells which can recognize these antigens are not deleted but enter into the state of anergy, since the tissue cells can not provide co-stimulatory activity. While clonal deletion and anergy as mechanisms of tolerance induction have been unequivocally demonstrated in experimental systems, a third mechanism, that of dominant suppression, is the subject of much controversy. In this model T cells play an active role in suppressing the activity of the other T cells which potentially mediate tissue damage (21,22,23).

The failure of the mechanisms responsible for self tolerance results in autoimmune disease. The underlying causes for this failure are not understood although infections in genetically susceptible individuals may trigger disease (9,24). Certainly autoreactive T cells have been demonstrated to be present in healthy individuals. In mice transgenic for an autoreactive TCR specific peptide of MBP bound to self MHC class II molecules, every T cell is potentially autoreactive and yet the mice remain healthy unless their T cells are deliberately activated (25,26). These T cells are considered to be in a state of immunological ignorance because the antigen to which

they respond is sequestered in the brain, an immunologically privileged site where the blood-brain barrier (BBB) prevents the entry of cells and molecules. In addition, the brain is immunologically silent, in that brain tissue does not normally express molecules which regulate (MHC) or mediate (cytokines) immune reactions. There is considerable evidence that activated T cells in small numbers routinely cross the BBB and patrol the brain. Only if these activated cells detect antigen to which they respond, do they take up residence and initiate an immune reaction (27). Paradoxically, therefore, an antigen which is sequestered in an immunologically privileged site can become the target for autoimmune attack.

Multiple sclerosis is an autoimmune disease of the central nervous system in which the myelin sheath surrounding neurons is destroyed. This can result in paralysis, sensory deficits, and visual problems. The etiology of the disease is unknown, although infectious agents and possibly environmental factors are suspected (9, 10). There is a strong genetic component to susceptibility to multiple sclerosis in that approximately 50-70% of MS patients carry the major histocompatibility complex (MHC) class HLA-DR2 allele compared to 20-30% in normal individuals (11,12). MS is characterized by a T cell and macrophage infiltrate in the brain and autoreactive, myelin-specific T cells have been isolated from MS patients, although T cells of the same specificity have been detected in normal individuals (13, 14, 15). The evidence, therefore, that MS is caused by pathogenic T cells is necessarily indirect, but the close resemblance which the characteristics of the disease bear to those of the murine model, experimental autoimmune encephalomyelitis (EAE) suggests that MS is indeed caused by an aberrant immune response mediated by T cells (16, 17, 18).

To date, treatment for MS and other autoimmune diseases has entailed non-specific immunosuppression using anti-inflammatory agents, or drugs which can block cell proliferation or probably depress proinflammatory cytokines. These treatments depend on how thoroughly the entire immune response is suppressed. These compounds also have serious side effects. The molecular insights that have been gained in recent years on T cell recognition and activation allows specific immunotherapies to

be designed to combat MS. For example, high affinity peptides can be synthesized which interact with MHC class II molecules and, prevent the binding of encephalogenic peptides, thereby, preventing the activation of pathogenic T cells (35). However, it is not clear at the present whether this approach can be developed into a therapeutic regiment, since it is difficult thus far to obtain effective concentrations of inhibitor peptides *in vivo* (36). In an alternate strategy, peptides which are analogs of encephalogenic sequences have been shown to antagonize the TCRs of antigen-specific T cells, rendering them unreactive, although the exact mechanism is at present unknown (37,38,39,40,41).

There are many methods to induce tolerance. The most effective method is to expose pathogenic T cells continuously to antigen in the absence of a co-stimulatory signal. Injection of synthetic peptides corresponding to the immunodominant determinants of MBP can block the progression and decrease the severity of EAE by inducing anergy in proliferating antigen-specific T cells (42). For example, repeated *iv.* injections of MBP protein into mice which are transgenic for a TCR specific for an encephalogenic MBP epitope (discussed above) have been shown to abrogate clinical pathological signs of EAE (2,43). The problem with this method is the requirement of a delivery system that can deliver high dose intravenous antigen at frequent intervals. EAE can be inhibited by inhalation; this has been achieved with a single encephalogenic peptide in PL/J mice (44). A list of encephalitogenic peptides of MBP, PLP, and MOG are listed in table 15.1.1 of chapter 15 of Current Protocols in Immunology, supplement 19 (73). This, too, has difficulty in the delivery system and one does not know what the bioavailability would be in a chronic human disease.

The effect of oral tolerance was first reported by Wells (1), who observed that guinea pigs fed hen egg protein were resistant to anaphylaxis challenge with the same protein. The apparent mechanism of oral tolerance has been the subject of numerous reports and appears to be associated with either antigen-specific stimulation of suppressive cells that secrete cytokines TGF β and IL-4 when administered at a low dose or deletion when administered at a high dose (2,3,4). Induction

of tolerance has been studied in a number of animal disease models for use in the treatment of autoimmune diseases, such as: multiple sclerosis by tolerizing with myelin basic protein (MBP) or proteolipid protein (PLP); rheumatoid arthritis by tolerizing with type II collagen; uveoretinitis by tolerizing with S-antigen or interphotoreceptor retinoid-binding protein; type I diabetes by tolerizing with insulin or glutamate decarboxylase; myasthenia gravis by tolerizing with acetylcholine receptor; and thyroiditis by tolerizing with thyroglobulin. Induction of tolerance is also currently being investigated for preventing transplant rejection by tolerizing with alloantigen or MHC peptide, and for prevention of allergic immune responses including allergic reactions to cats and bee stings by tolerizing with Fel d1 or PLA-2 peptides, respectively (5,6). For current review of the field see proceedings of the conference "Oral Tolerance: Mechanisms and Applications" (74). Induction of tolerance to autoantigens has recently been applied in human clinical trials for multiple sclerosis, rheumatoid arthritis, uveoretinitis, and type I diabetes (5). The results of two of these studies showed that a small portion of patients responded favorably to the treatment that consisted of feeding bovine derived MBP or bovine derived type II collagen for the treatment of multiple sclerosis or rheumatoid arthritis, respectively (7,8). This type of treatment is appealing because of the lack of toxicity and low incidence of adverse side effects observed.

Other methods of tolerance restoration which have been tested in EAE include intrathymic injection of MBP and intraperitoneal injection of synthetic peptide (45, 46). Both of these are problematic for development into a human treatment because of the method of delivery. Recent phase I/II clinical trials using a synthetic peptide containing the immunodominant B-cell and T-cell epitope of MBP pMBP86-95 or pMBP82-98 administered intrathecally to MS patients showed some effect for the prevention and/or treatment of acute relapses of MS, whereas intravenous administration showed a failure to prevent relapses (70). The administration of oral bovine myelin in human MS phase I/II trial would appear to be the most promising method. The initial study showed no adverse toxicity or side effects and

showed limited success in a subpopulation of treatment patients with specific MHC alleles, which suggests that oral tolerance, thus far, may have limited efficacy. A continuation of the phase I/II oral myelin trial were examined were 17 relapsing-remitting MS patients received 300 mg of bovine myelin daily for two years. This study demonstrated no Th1 sensitization after prolonged oral antigen administration in MS patients; instead, MBP- and PLP-reactive TGF- β 1-secreting cells were induced (60).

Additionally, there are many questions about oral tolerance that remain unanswered even in EAE. There are also questions raised about bioavailability of an oral preparation in order to have a continuous antigen TCR contact that will delete pathogenic T cells. There are clearly low dose and high dose effects, and Michison in 1964 wrote of high zone tolerance (47). A recent paper by Chen et al. discusses high and low dose tolerance and the dependence on dosage frequency of feeding (48). A pilot trial using oral administration of bovine myelin on MS patients suggests that this treatment may be efficacious for some but not all individuals (7). In summary, the question of the delivery of tolerizing antigens is far from settled.

Previous animal studies have shown that feeding of species specific MBP results in favorable results in treating EAE. However, feeding heterologous MBP also exhibited beneficial results. In human Phase III clinical trials, purified fractions of bovine myelin were administered orally to MS patients (60). Preliminary results from this study did not demonstrate efficacy (72) nor did results of similar Phase III trials in which purified fractions of chicken type II collagen were administered orally to rheumatoid arthritis patients also failed to show a difference between treated and placebo groups. (63,72). Unfortunately, there are questions arising from the restricted response of the patients. There are problems relating to the bioavailability of the antigens related to humans, the sequence differences between bovine and human myelin (there is only 84% homology), and there is a potential threat of bovine spongiform encephalopathy that may result from feeding bovine brain to humans. Recent studies conducted on transgenic mice expressing chimeric human/mouse HLA-DR1 were immunized with human type II collagen isolated from sternal cartilage harvested from human

donors (71). These mice developed severe autoimmune arthritis accompanied by T- and B-cell responses to human type II collagen. The dominant T-cell response was focused on a dominant determinant contained within huCII(259-273). This provides a very useful model in which to study therapies for rheumatoid arthritis, but again the bioavailability of human type II collagen is an issue.

Transgenic plants have been used to produce a variety of heterologous or foreign proteins. Some examples to date are the production of interferon in tobacco (Goodman et al., 1990, (64), enkephalins in tobacco, *Brassica napus* and *Abutilon thaliana* (Vendkerchove et al., 1989), human serum albumin in tobacco and potato (Sijmons et al., 1990 (66), antibodies in tobacco (Hiatt et al., 1990 (67) and hepatitis B antigen (Mason et al., 1992 (68). While there have been suggestions to use transgenic plants for producing a variety of proteins and vaccine antigens, only recently has there been demonstration of expression of murine GAD67 in transgenic tobacco or potato plants for use in the prevention of diabetes in NOD mouse model (69). In order to overcome reduced immunological activity and poor solubility of GAD expressed in mammalian or microbial expression systems, transgenic tobacco expressing the murine GAD67 protein were found to retain its T-cell recognition and stimulator specificity. This demonstrates the potential use of transgenic plants expressing tolerogenic or autoantigens for the induction of tolerance in susceptible animal models, and that oral delivery of the autoantigens or allergens expressed in the plants could be effective to protect or prevent autoimmune diseases or anaphylaxis challenge in animals including humans. In the case of vaccine antigens produced in transgenic potato plants, the expression levels were found to be largely affected by the site of DNA integration into the plant genome which occurs in a random fashion. RNA expression in tuber material appears to be more stable than in the leaves. However, there have not been developed plants which have been generated to express human tolerizing proteins for human autoimmune diseases.

Thus, there is a need for delivering autoimmune antigens or allergens to animals including humans and presenting large doses of the antigens to the body in such a way that they are

not destroyed by digestive juices, liver enzymes, and the like. There is a need to deliver autoimmune antigens or allergens by directly feeding transgenic plants, plant organs or seeds containing the tolerogenic antigen to patients in need thereof.

- 5 There is a need to provide tolerizing antigens in compositions derived from transgenic plants, plant organs or seeds. The tolerizing antigen can be used as an orally administered pharmaceutical in the transgenic plant, plant organ or seed directly, or can be extracted and purified for other uses.

SUMMARY OF THE INVENTION

The present invention tolerizes animals, especially humans, against immune-mediated diseases and conditions, such as autoimmune diseases and allergic reactions, by feeding to the
5 animal a tolerizing dose of a suitable tolerogenic antigen. This antigen is administered in the form of a transgenic plant, or a plant part or plant product which retains the antigen in a tolerizing amount and form, the plant having been genetically engineered to express the antigen. Preferably, the plant is
10 itself edible.

In one embodiment, the tolerogenic antigen is an autoimmune antigen and provides for protection against autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, diabetes, and the like.

15 In another embodiment, the tolerogenic antigen is an allergen, and provides tolerance induction for the prevention of anaphylactic reactions to the tolerizing antigen. In a related embodiment, the tolerogenic antigen is an MHC alloantigen for protection against transplant rejection.

20 The invention provides for preparation of food (including a beverage) comprising or derived from the transgenic plants of the present invention. The food may comprise a transgenic plant, plant organ or plant seed which includes an expression cassette to the invention, along with nutritionally acceptable
25 adjuvants such as flavorings, seasonings, and carriers. The food may incorporate the plant, plant organs or seeds thereof in a raw or processed state.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The transgenic plants which produce tolerogenic antigens are obtained by introducing into the plant an expression
5 construct comprising a DNA sequence encoding a tolerogenic antigen and regulatory sequences capable of directing the expression of the autoimmune or tolerogenic antigen in the plant, plant organ, or seeds. The expression construct provides for the stable transformation of the plants. The transgenic
10 plants, plant organs, seeds or other plant-derived food (including beverage) product containing the antigen may be used as a practical delivery system of the antigen to a patient afflicted with an autoimmune disease or a patient afflicted with an allergy. Alternatively or additionally, the antigen can be
15 isolated and administered to patients in the form of a pharmaceutical composition to stimulate active or passive suppression. The tolerogenic antigen can also be isolated and purified for use in diagnostic assays.

Tolerogenic Antigens

20 Tolerogenic antigens, as defined in the context of the present invention, include both autoimmune antigens and antigens involved in eliciting specific allergic reactions (allergens). Such that induction of tolerance to the antigen prevents or otherwise inhibits the development of an autoimmune disease or
25 of a detrimental allergic response with the same (or cross-reactive) antigen. According to the present invention, transgenic plants are generated which express tolerogenic antigens (tolerogens). These antigens are used to treat patients suffering from autoimmune diseases or allergic
30 reactions, or to prevent such conditions. The tolerogenic antigens can be administered orally, in the form of the plant, a plant part, or a food or beverage derived from said plant.

The term "autoimmune antigen" and "allergen" include
35 molecules which are immunologically cross-reactive with the native autoimmune antigen or allergen, and capable of acting as tolerogenic antigens specifically suppressing the response to the native antigens.

The methods and compositions are directed towards treating and protecting humans as well as animals, including domestic animals. Protection against disease includes amelioration to the symptoms of the disease, decrease in mortality and morbidity, or decrease in sensitivity to the antigen. While it is not meant to be a limitation of the invention, it is believed that the act of chewing the transgenic plant or food containing the transgenic plant can result in delivering the tolerogenic antigen both orally and through the oral mucosa, including the tonsils. In addition, the administration of a large dose of transgenic plant material can permit the passage of the autoimmune or tolerogenic antigen containing material to the intestinal tract without inactivation of the tolerogenic antigen.

Treatment of patients with the autoantigens produced according to the present invention should result in the amelioration of symptoms, decrease in morbidity, and prevention of further disease development. The autoimmune diseases treated by the method of the present invention include but are not limited to diabetes mellitus type I, multiple sclerosis, rheumatoid arthritis, myasthenia gravis, uveoretinitis, and thyroiditis. Known autoimmune antigens include human myelin basic protein, type II collagen, proteolipid protein, S-antigen, interphotoreceptor binding protein, insulin, glutamate decarboxylase, acetylcholine receptor, and thyroglobulin.

Known allergens for the prevention of allergic reactions or hypersensitivity to the allergens, by the method of the present invention, include but are not limited to the expression of allergens of feline FEL di, bee venom PLA-2, mite allergen 70-80 kD, for the prevention of allergic reactions to the defined allergens, or MHC alloantigen for the prevention of transplant rejection.

Certain tolerizing proteins for these diseases are known, and these tolerizing proteins can readily be generated in transgenic plants according to the present invention and used to treat patients suffering from these diseases. Amino acid sequences are available from GenBank, as set forth below. The native DNA sequence may also be retrieved from GenBank, or a DNA sequence may be designed de novo with the aid of the Genetic

Code.

Disease	Protein Fed	Gen Bank Accession
Multiple sclerosis	Human Myelin basic protein (MBP)	M13577
5	Human myelin proteolipid protein (PLP)	M54927
	Human myelin-oligodendrocyte glycoprotein (MOG)	X74511
Rheumatoid arthritis	Human Type II collagen	L10347
10 Uveoretinitis	Human S-antigen	X12453
	Human Interphotoreceptor retinoid-binding protein (IRBP)	M22453
Type I diabetes	Human Insulin	J00265
15	Human Glutamate decarboxylase (GAD)	M81882
Myasthenia gravis	Human heat shock protein (HSP)	M11717
Thyroiditis	Human Acetylcholine Receptor (AChR)	S77094
Transplantation	Human Thyroglobulin	X05615
	Alloantigen	
20	Human MHC HLA protein allele	M32317;
Allergic Reaction to:	B7 (MHC)	M35444
Animal dander	Feline domesticus major allergen (Fel d1)	M74952
Bee venom	Honeybee Phospholipase A2 (PLA-2)	X16709
25	Dust mite	Mite Allergen (Der p I)
		U11695;
		S66377;
		Y00641
Dust mite	Mite allergen (Der f I)	D10448;
		S70378

30 If the amino acid sequence of a protein of interest is not published, it may be determined by (a) purifying and sequencing the protein, or (b) isolating the corresponding genomic or cDNA, using a primer or hybridization probe, sequencing the isolated DNA, and inferring the a.a. sequence. Suitable probes or

35 primers may be DNAs encoding related proteins (use relaxed hybridization conditions suitable for DNAs with 50-70% identity), or DNAs encoding one or more known partial a.a.

sequence(s) of the protein of interest (if this is a mixed probe, use stringent conditions, T of 5-10°C below T_m; if the probe is a unique probe based on codon preferences or containing inosine, use relaxed conditions).

5 Transgenic Expression

The DNA sequence encoding a tolerogenic antigen may be a genomic DNA, a cDNA, a synthetic DNA, or a combination thereof. If a synthetic DNA, it may be designed for compatibility with the codon preferences of the host plant, to avoid formation of secondary structures, to facilitate later manipulation, etc. The coding sequence is operably linked to transcriptional and translational control regions conducive to expression in the host plant. Transcriptional and translational control regions include promoters, enhancers, cis regulatory elements, polyadenylation sequences, transcriptional and translational initiation regions, and transcriptional termination sequences.

The promoters are preferably those that provide for a sufficient level of expression of a heterologous gene to provide for enough tolerogenic antigen to treat a patient by oral administration. The promoters are those that are functional in plants and preferably provide for a level of heterologous gene expression about the same as or greater than that provided by the 35S cauliflower mosaic virus (35S CaMV) (cp. USP 5,623,066) promoter in the particular plant type. The especially preferred promoters are those that provide for a level of gene expression of about 0.1% to 10% of the total cell protein. Promoters can be inducible or constitutive, and they can be expressed in all tissues or only in specific tissues. Specific examples of promoters include the 35S CaMV promoter, the nopaline synthase promoter, the chlorophyll A/B binding promoter, the phaseolin promoter, the waxy promoter, and napin promoter, the ubiquitin promoter, the (AOCs)3AMAS Pmas promoter and the following:

Chimeric GUS Genes and Expression Levels with Various Promoters

PROMOTER	TISSUE	AV. GUS LEVEL NMOL MUG/MG	REFERENCE
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PROTEIN-MIN

5	T7 RNA polymerase	tobacco leaf (chloroplast)	20,000 (20-30% of total soluble protein)	McBride KE, Schaaf DJ, Daley M, Stalker DM (1994) Proc. Natl. Acad. Sci. USA 91, 7301-7305.
10	B. napus napin	tobacco seed ABA induced	400 1200	Jiang L Abrams SR, Kermode AR (1996) Plant Physiology 110, 1135- 1144.
15	Potato ubi7	potato leaves tuber peel	100 10	Garbarino JE, JE, Oosumi T, Belknap WR (1995) Plant Physiology 109, 1371-1378.
20	P. vulgaris phaseolin	tobacco seed	83	Frisch DA, van der Geest AHM, Dias K, Hall TC (1995) Plant Journal 7, 503-512.
25	Potato Sus4	potato tuber	58	Fu H, Kim SY, Park WD (1995) Plant Cell 7, 1387-1394.
30	Potato ubi3	potato tuber	38	Garbarino JE, Belknap WR (1994) Plant Mol. Biol. 24, 119-127.
35	Potato patatin	potato tuber	23	Fu H, Kim SY, Park WD (1995) Plant Cell 7, 1387-1394.
40	(ocs) ₃ mas	tobacco leaves	22	Ni M, Cui D, Einstein J, Narasimhulu, Vergara CE, Gelvin SB (1995) Plant Journal 7, 661-676.
45	Tobacco SAR +CaMV 35S GUS (pBI101)	tobacco leaves	20	Allen GC, Hall G, Michalowski, Newman W, Spiker S, Weissinger AK, Thompson WF (1996) Plant Cell 8, 899-913.
50	CaMV dbl 35S	tobacco leaves	0.85	Ni M, Cui D, Einstein J, Narasimhulu, Vergara CE,

			Gelvin SB (1995) Plant Journal 7, 661-676.
5	CaMV 35S (pBI101)	tobacco leaves	0.142
			Ni M, Cui D, Einstein J, Narasimhulu, Vergara CE, Gelvin SB (1995) Plant Journal 7, 661-676.
10	Rice waxy	tobacco seeds	0.04
			Hirano HY, Tabayashi N, Matsumura T, Tanida M, Komeda Y, Sano Y (1995) Plant Cell Physiol. 36, 37-44.

See, e.g., WO92/01042; WO90/02189; USP 5,182,200.

Transcriptional and translational control regions are typically present in expression vectors. Preferably, expression
 15 vectors are selected for compatibility and stability in the type of plant cell to be transformed. Some expression vectors including promoters and the 3' regulatory regions are commercially available such as CaMV vector, binary vectors such as pBI101 (Available from Clone Tech, Palo Alto, CA). Expression
 20 vectors can also include those used in amplification and selecting steps such as the baculovirus vector, or phage lambda, or other plasmid vectors useful in amplification and cloning of DNA sequences.

Once an expression cassette is formed and subcloned into an
 25 appropriate vector system, it can be transformed into suitable host cells. Suitable host cells include bacteria such as *E. coli*, *Agrobacterium tumefaciens*, Sf9 insect cells for baculovirus and plant cells or tissues such as corn suspension cultures, wheat callus suspension cultures, rice protoplast, soy
 30 bean tissue, sunflower tissue, alfalfa tissue, green bean tissue, and other edible plant cells and tissue. The expression system and vector selected is one that is compatible with and stable in the selected host cell. For plant cell transformation, vectors are preferably selected to maximize
 35 stable integration of the foreign DNA into the plant cell genome.

Methods of transforming cells depend on the type of host cell selected. For bacterial host cells, methods of transformation

include the freeze/thaw method, calcium phosphate precipitation, and electroporation. For plant cell transformation, preferred methods of transformation include *Agrobacterium* mediated transformation, direct transformation of protoplast using electroporation, or direct transfer into protoplast or plant tissue using microparticle bombardment, or combinations of these methods.

Among the plant cells and tissues to be transformed include those plants useful as food plants, including alfalfa sprouts, barley, beans, corn, flax, lentils, sorghum, mustard and rapeseed, oats, rye, *Perilla*, sesame, safflower, soybeans, sugar beets, sunflowers, spinach, tomatoes, potatoes, bananas, peanuts, broccoli, carrots and wheat and those listed in Table A of PCT publication number WO 95/23229, the entire contents of which are hereby incorporated by reference. The preferred plants are those which can be readily incorporated into a conventional diet for oral administration. The plants may be monocots or dicots.

The tolerogenic antigen protein may be expressed in the seed of seed-producing plants (cp. WO91/13993; EP 295,959; EP 255,378; USP 5,504,200; USP 5,215,912), such as sunflower, which can be used as a source of food either whole or in the form of flour or oil. In plants where the leaves are eaten, such as spinach, constitutive expression is preferred. In plants where the plant organs, such as tomato, are eaten, tissue specific expression is preferred.

Transformed plant cells are cultured under conditions that select for those cells having the expression cassette, typically by selecting for those cells that exhibit antibiotic resistance. Antibiotic resistance genes are typically used as selectable marker genes. The transformed cells are also grown under conditions that favor regeneration of the cells and/or tissue into plants. These techniques are known to those of skill in the art. The presence of the desired DNA sequence coding for at least one tolerogenic antigen in the plant cells or tissues can be determined by hybridization with a probe or by detecting expression by assaying for the presence of the tolerogenic antigen and other like assays.

Once transgenic plants are obtained, they can be grown under

appropriate field conditions until they produce seed. The presence of the DNA sequence encoding for the autoimmune or tolerogenic antigen and expression of the autoimmune or tolerogenic antigen in the transgenic plant can be determined and quantitated by conventional techniques. An expression cassette encoding at least one tolerogenic antigen is preferably stably integrated into the plant cell genome. Stable integration of an expression cassette into a plant cell genome may be established when found in three successive generations. Methods for detection of expression of a protein encoded for by the inserted DNA include SDS-PAGE electrophoresis, Western blot, ELISA and other methods known in the art. The presence of the DNA sequence encoding for the tolerogenic antigen in the plant genome or chromosomal material can be verified and the copy number can be quantitated using hybridization methods known to those skilled in the art. The level of gene expression can be quantitated using quantitative Northern blots or by measuring the amount of specific mRNA synthesis by quantitative PCR. Transgenic plants that express the most tolerogenic antigen as a percentage of the total plant cell protein are preferably selected for further propagation. These plants preferably express the tolerogenic antigen within the range of about 0.1 to about 10% of the total plant protein.

Transgenic plants can be crossed with known parental strains and the progeny plants evaluated for the presence of a DNA sequence encoding the tolerogenic antigen and/or expression of the tolerogenic antigen. The especially preferred transgenic plants of the present invention are those that can transmit the DNA sequence encoding the tolerogenic antigen to the next generation of plants. The term "transgenic plants" includes not only the originally engineered plants, but also progeny who retain the transgene in functional form. It also includes hybrids of transgenic and nontransgenic plants if the hybrids express the transgene.

Transgenic seed can be collected from transgenic plants and the level of gene expression of the tolerogenic antigen in the seed can be determined as described above. The level of gene expression of the tolerogenic antigen in the seed is preferably that amount that provides for effective prevention, inhibition

or treatment of the particular autoimmune disease or allergic reaction treated.

Delivery of Tolerogenic Antigen to the Patient

The transgenic plants of the present invention can be incorporated into a patient's diet in the form of a food. The term "food" or "food product" as used herein, is intended to include articles defined as "food" by the Federal Food, Drug and Cosmetic Act, Sec. 201(g), i.e., "(1) articles used for food or drink for man or other animals, (2) chewing gum, and (3) articles used for components of any other such article". It also includes "food additives", as defined by Section 201(s) of that act. The food may be a fresh, partially processed, or fully processed food, including a canned, frozen or dry food, or a beverage. The USFDA has standards of identity for, e.g., frozen desserts (part 135), bakery products (part 136), cereal flours (part 137), macaroni and noodle products (part 139), canned fruits (part 145), canned fruit juices (part 146), fruit butters, jellies, preserves and related products (part 150), fruit pies (part 152), canned vegetables (part 155), vegetable juices (part 156), frozen vegetables (part 158), cacao products (part 163), tree nut and peanut products (part 164), nonalcoholic beverages (part 165), sweeteners and table syrups (part 168), and food dressings and flavorings (part 169), which could derived in whole or part from a transgenic plant. However, the food products are not limited to standardized foods. The whole plant, a plant part, or a product derived from the plant, e.g., a juice, soup, candy bar, cake, or cracker, may be fed to the patient. The transgenic plants or parts thereof can be incorporated in standard recipes using conventional food or beverage preparation techniques.

The amount of the transgenic plant, plant part or other plant derived material added to the diet is that amount that provides sufficient tolerogenic antigen to prevent, inhibit, treat or otherwise protect against the autoimmune disease or allergic reaction. The amount of tolerogenic antigen administered in the diet will vary depending upon the type of autoimmune disease or allergic reaction, the frequency of administration, the weight of the patient, and the frequency of administration. The

appropriate quantity of the tolerogenic antigen contained in the plant materials can be determined using standard methodology. Once the amount of tolerogenic antigen in the food is determined, the amount of the food to be included in the diet
5 can be calculated.

The food can be administered by incorporating the plant, plant organs and/or seed material into a patient's daily diet, either daily or on another periodic basis.

Other tolerizing agents, such as the histamine derivatives
10 of WO93/13772, may, if orally compatible, likewise be incorporated into the food or beverage.

Transgenic plant or plant parts or other plant-derived food products, containing a tolerogenic antigen provide a low cost, easy to administer composition for treating autoimmune disease
15 or allergic reaction.

In another embodiment of the invention, a pharmaceutical composition may be prepared. The pharmaceutical composition may include an extract including the tolerogenic antigen from a transgenic plant which provides for protection from the
20 autoimmune disease or allergic reaction for which the antigen is specific. According to the present invention, oral administration of a pharmaceutical composition of the present invention can be used to treat a patient suffering from an autoimmune disease or allergic reaction as well as to prevent
25 development of an autoimmune diseases or allergic reaction. The pharmaceutical composition of the invention is preferably administered orally, such as by preparing the transgenic plant as part of the diet of the patient. An effective oral dose of the pharmaceutical composition can be readily determined by one
30 skilled in the art. For protection against allergic reactions, intranasal administration may be appropriate. However, any standard route, e.g., intravenous, subcutaneous or intramuscular, may be employed.

EXAMPLES

35 Examples 2-7 are based on WO94/02620 (USP 5,563,055) and WO97/10347.

Example 1:

Construction of Plant Vectors Containing MBP

Plant vectors were constructed which contain huMBP by isolating huMBP cDNA from human brain Quick-Clone cDNA (Clontech, Palo Alto, CA) using polymerase chain reaction and MBP specific oligomers: 5' oligomer 5'TCCCCCGGGATGGCATCACAGAAGAGACCC3' containing the restriction enzyme site for SmaI and 3' oligomer 5'GCTCTAGAGCTCTCAGCGTCTCGCCATGGGAGA3' containing the restriction enzyme sites for SacI and XbaI. The 516 base pair PCR fragment coding for human MBP was digested with SacI and XbaI and cloned into the SacI and XbaI sites of the baculovirus transfer expression vector pVL1393 (PharMingen, San Diego, CA) The clone MBP1393 containing the huMBP was isolated and confirmed by DNA sequencing. This clone was used to generate recombinant huMBP baculovirus. Baculovirus expressed huMBP is analyzed for reactivity with a panel of MBP specific monoclonal antibodies obtained from (Biogenesis, Inc. Sandown, NH). This material is used as a positive control in western and ELISA analysis of MBP expression in transgenic plants.

The SmaI/SacI fragment containing the huMBP fragment was subcloned from MBP1393 into the SacI and SmaI sites of plant expression vectors IBT140 and IBT110, obtained from Dr. Hugh Mason, Boyce Thompson Institute for Plant Research. The IBT110 vector is described in Tariq et al. (49) and the IBT140 vector is described in Mason et al (50). The IBT110 expression cassette contains a dual enhancer 35S promoter, whereas the IBT140 expression cassette contains a patatin promoter. The 35S promoter is a constitutive promoter, whereas the patatin promoter is tuber specific. Both expression vectors contain an NTP II cassette for selection of kanamycin resistance, the TEV leader sequence at the 5' end of the inserted gene to enhance translation, the soybean vspB terminator at the 3' end of the inserted gene, and T-DNA boarder sequences for transfer of the foreign DNA into the plant genomic DNA. The recombinant MBP110 and MBP140 clones were isolated and confirmed by DNA sequence analysis. The huMBP fragment was modified to containing a 6xhis-tag-peptide sequence at the carboxy terminus to facilitate concentration or screening of the recombinant proteins expressed in transgenic plants. The huMBP-6xhis was generated from clone MBP1393 using PCR and the following oligomers: 5' oligomer

5'AGAGAGGCTGAAGCTAGATCTGGATCCA ATGGCGTCACAGAAGAGACCCTCC3'
containing the restriction enzyme sites for BglII and BamHI and
3' oligomer

5'GCCGCCGCGGCTCGAGAGCTCTCAATGATGATGATGATGATGGGTACCGCGTCTAGCCA
5 TGGGTGATCCAGA3' containing the restriction enzyme sites for SacI
and KpnI. The PCR fragment digested with SacI and BglII was
subcloned into the BamHI and SacI sites of IBT110 and IBT140
plant expression vectors.

Plant vectors were constructed which contain 833 base pair
10 huPLP were constructed in a similar fashion from human brain
Quick-Clone cDNA (Clontech, Palo Alto, CA) using polymerase
chain reaction and huPLP specific oligomers: 5' oligomer
5'CGCATGGATCCGTTAGAGTGCTGTGCAAGATGTCT3' containing the
restriction enzyme site BamHI and 3' oligo
15 5'TCGCGAGCTCGGGATCAGAAC3' containing the restriction enzyme site
SacI. The PCR fragment was cloned into the pGemT vector
(Promega, Corp. Madison, WI).

Generation of transgenic plants expressing huMBP or
huMBP6xhis are described below as MBP constructs and
20 transformants.

Agrobacterium mediated transformation of potato plants with MBP Vectors

Recombinant MBP110 and MBP140 clones were isolated from *E.*
coli and transferred to *Agrobacterium tumefaciens* strain
25 LBA4404. The transformed *Agrobacterium* strains carrying either
the MBP140 or MBP110 were used to transform potato plants
(*Solanum tuberosum*, variety "Frito-Lay" 1607) by leaf-disc
co-cultivation methods (49, 51). Kanamycin-resistant calli are
transferred to shoot-induction media, and individual shoots are
30 isolated from the calli and transferred to rooting media.
Primary transformants are screened for MBP expression by RNA
dot/blot and ELISA analysis using MBP specific DNA probes or MBP
specific monoclonal antibodies for huMBP containing clones or
monoclonal antibodies to 6xhis (Clontech Laboratories, Palo
35 Alto, CA) for huMBP6xhis containing constructs. Positive clones
are further characterized by Northern and Western analysis.
Both leaf and tuber tissue is screened for MBP110 transformants,

whereas only tuber tissue is screened for MBP140 transformants. Under normal conditions the patatin protein is not expressed in leaves or stem tissue of potato. However, in tuber tissue it accounts for approximately 40% of the total soluble protein
5 (52).

MBP positive transformants are propagated vegetatively by stem tissue culture. After multiplying, the transformants are transferred to soil and grown in a plant growth chamber for production of tuber material for animal feeding. Previous
10 reports indicate a mouse will consume 5 grams of tuber material within a 2 to 6 hour time period (49). The amount of MBP per gram of tuber is quantitated prior to feeding. Low yields of huMBP6xhis can be concentrated using TALON metal affinity resin (Clontech Laboratories, Palo Alto, CA).

15 Example 2

Formation of an Expression Cassette for Expressing Human Type II Collagen in Corn

An expression cassette for expression of Type II collagen in corn, which is useful for treating rheumatoid arthritis, can be
20 formed as follows:

The DNA sequence coding for human type II collagen is isolated from cDNA obtained from human chondrocytes (61), using polymerase chain reaction and hu-type II collagen specific oligomers. The plasmid contains the T6 ubiquitin promoter and
25 intron with a PinII termination sequence. The gene can be blunt end ligated into the sites or additional cloning sites could be inserted to make this compatible with other genes that provide for constitutive expression of a heterologous gene under control of the ubiquitin promoter.

30 Briefly, cDNA can be prepared from mRNA using reverse transcriptase and oligo dT primers or a specific primer designed from the known DNA sequence (GenBank accession number L10347).

Double stranded cDNA can be dC-tailed using terminal transferase and annealed to a dG-tailed restriction endonuclease
35 cleaved vector, or cDNA can be amplified using PCR and specific primers which incorporate restriction enzyme sites at the 5' ends to facilitated site directed cloning of the PCR product encoding the hu-type II collagen into the compatibility cleaved

vector. The vectors can be introduced into a bacterial host cell, and transformants carrying antigen inserts can be identified using probes designed for the known DNA sequence or by using antibodies specific for the type II collagen.

5 Once the DNA sequence coding for type II collagen is isolated, it can be subcloned into plant expression vectors such as the modified vector sites so that the expression of this DNA sequence is under control of the ubiquitin promoter. Plasmids including the DNA sequence coding the type II collagen can be
10 selected by examining the restriction digest patterns from plasmids that were isolated from cells growing on ampicillin. This sequence is confirmed by DNA sequencing.

Example 3

Preparation of Transgenic Corn Having an Expression Cassette 15 Coding for Human Type II Collagen

Once formed, a vector carrying a DNA sequence coding for type II collagen under control of a promoter functional in the plant can be used to form transgenic corn plants. A method for formation of transgenic corn plants is described in European
20 Patent Application No. 0 442 174A1, the entire contents of which are hereby incorporated by reference. A brief description of that methodology follows.

A vector carrying a DNA sequence coding for type II collagen formed as described in Example 2 can be introduced into corn
25 tissue suspension cells by microparticle bombardment. In addition, a construct containing a 35S expression cassette can be cotransformed with the type II collagen to allow for easy selection of transformed plants. The 35S cassette is disclosed in Gordon-Kamm et al., The Plant Cell 2:603-18 (1990). 35S
30 contains the BAR gene, which has been shown to impart resistance to glufosinate selective agents in cells.

Preferably, germ cells are used, including those derived from a meristem of immature embryos. Suspension cell lines are also available to generate embryogenic suspension cultures. For
35 example, embryogenic suspension cultures can be derived from type II embryogenic culture according to the method of Green et al., Molecular Genetics of Plants and Animals, editors Downey et al., Academic Press, NY 20, 147 (1983). The callus can be

initiated from maize inbreds. Suspension cultivars of the cultivar "Black Mexican Sweet" (BMS) can be obtained from Stanford University. The cultures can be maintained in Murashige and Skoog (MS) medium as described in Murashige et al., Physio. Plant 15: 453-497 (1962) supplemented with 2,4-dichlorophenoxyacetic acid at 2 mg/L and sucrose at 30 g/L. The suspension cultures are passed through a 710 micron sieve seven days prior to the experiment and filtrate can be maintained in MS medium. In preparation of microparticle bombardment, cells are harvested from the suspension culture by vacuum filtration on a Buchner funnel (Whatman No. 614). Alternatively, callus cells can be passed through a sieve and used for bombardment.

Prior to the microparticle bombardment, a 100 ml (fresh weight) of cells are placed into a 3.3 cm petri plate. The cells are dispersed in 0.4 mL fresh culture medium to form a thin layer of cells. The uncovered petri plate is placed in the sample chamber of a particle gun device manufactured by Biolistics Inc., Geneva, NY. A vacuum pump is used to reduce the pressure in the chamber to 0.1 atmosphere to reduce deceleration of the microparticles by air friction. The cells are bombarded with tungsten particles having an average diameter of about 1.2 microns, obtained from GTE Sylvania Precision Materials Group, Towanda, PA. The microparticles have a DNA loading consisting of equal mixtures of the selectable and nonselectable plasmids. The DNA is applied by adding 5 microliters of 0.1 g% suspension of 50 mg of tungsten particles per mL distilled water in a 1.5 mL Eppendorf tube. Particles become agglomerated and settle.

Cultures of transformed plant cells containing the foreign gene are cultivated for 4-8 weeks in 560R medium (N6-based medium with 3 mg/mL of bialaphos). After this time, only cells that received the BAR gene are able to proliferate. These events are rescued and identified as transformants. The putative transformants are then tested for the presence of integration of Type II Collagen DNA by PCR. Transient expression of the DNA sequence coding for the type II collagen at 24-72 hours after bombardment can be detected using Western blots, ELISA and antibodies to the type II collagen.

Embryo formation can then be induced from the embryogenic cultures to the stage of maturing and germination into plants. A two culture medium sequence is used to germinate somatic embryos observed on callus maintenance medium. Callus is transferred first to a culture medium (maturation medium) which, includes 5.0 mg/L indoleacetic acid. The callus culture remains on this medium for ten to fourteen days while callus proliferation continues at a slower rate. At this culture stage, it is important that the amount of callus started on the culture medium not be too large, or fewer plants will be recovered per unit mass of material. Especially preferred is an amount of 50 mg of callus per plate.

Toward the end of this culture phase, observation under a dissecting microscope often indicates somatic embryos have begun germinating, although they are white in color because this culture phase is conducted in darkness. Following this first culture phase, callus is transferred from "maturation" medium to a second culture medium which further promotes germination of the somatic embryos into a plantlet. This culture medium has a reduced level of IAA versus the first culture medium, preferably a concentration of about 1 mg/L. At this point, the cultures are placed into the light. Germinating somatic embryos are characterized by a green shoot which elongates often with a connecting root access. Somatic embryos germinate in about ten days and are then transferred to medium in a culture tube (150 x 25 mm) for an additional ten to fourteen days. At this time, the plants are about 7-10 cm tall, and are of sufficient size and vigor to be hardened off to greenhouse conditions.

To harden off regenerated plants, plants are removed from the sterile containers and solidified agar medium is rinsed off the roots. The plantlets are placed in a commercial potting mix in a growth chamber with a misting device which maintains the relative humidity near 100% without excessively wetting the plant roots. Approximately three or four weeks are required in the misting chamber before the plants are robust enough for transplantation into pots or into field conditions. At this point, many plantlets, especially those regenerated from short term callus cultures, will grow at a rate into a size similar to seed derived plants. Ten to fourteen days after pollination,

the plants are checked for seed set. If there is seed, the plants are then placed into a holding area in the greenhouse to mature and dry down. Harvesting is typically performed six to eight weeks after pollination.

5 This methodology has been used successfully to regenerate corn plants expressing the chloramphenicol acetotransferase gene under control of the 35S cauliflower mosaic virus (35S CaMV) promoter, as well as many other sized genes. Direct introduction of foreign DNA into suspension culture or tissues
10 of monocot plants has been used successfully for regenerating transgenic monocot plants such as corn, wheat, rice and the like.

Example 4

Formation of Transgenic corn Seeds carrying an Expression 15 Cassette Coding for Human Type II Collagen

The DNA sequence coding for type II collagen can be inserted into an expression cassette under control of the waxy promoter for seed specific expression. A cassette is present in a vector that has the waxy regulatory sequences and a heterologous gene
20 encoding sequence, and can be inserted into appropriate restriction enzyme sites. Alternatively, the heterologous gene can be blunt end ligated or additional cloning sites can be added to make them compatible with the coding sequence of the heterologous gene.

25 Transgenic corn containing the DNA sequence coding for human type II collagen can be obtained as described in Example 3 or as described in US patent 5,591,616, the entire contents of which are hereby incorporated by reference. A brief description of that methodology follows.

30 This method of transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with bacterium belonging to genus *Agrobacterium* containing a desired gene.

This DNA sequence can be inserted into the multiple cloning
35 site in the plasmid using standard methods. A plasmid including a DNA sequence coding for human type II collagen under control of a seed specific promoter can be selected and isolated by examining the restriction patterns of the recombinant plasmid

and sequencing.

Corn cells are transformed by microparticle bombardment as describe in Example 3. Transformed cells containing a DNA sequence coding for human type II collagen can be identified and selected by PCR. Transgenic corn plants and seeds can be regenerated as described in Example 3. Expression of human type II collagen in seeds can be confirmed and quantitated by ELISA or Western blot analysis. Stability of the expression of the human type II collagen can be evaluated by these same methods over successive generations.

Example 5

Formation of an Expression Cassette Encoding S-antigen and/or Interphotoreceptor retinoid-binding Protein

An expression cassette can be formed for expression of S-antigen and/or interphotoreceptor retinoid-binding protein under control of the promoter for the seed storage protein phaseolin. The expression cassette can be formed with a DNA sequence encoding S-antigen and a DNA sequence encoding interphotoreceptor retinoid-binding protein under control of the single promoter to form a dicistronic construct, or each DNA sequence can be placed under control of its own promoter but the same promoter. The expression cassette is present in a vector such as pPHI4852.

Plasmid is prepared by linking the phaseolin upstream regulator region adjacent to the downstream region of the phaseolin gene, but not including the coding sequence of the gene itself. Plasmid has restriction enzyme sites that can be used to insert heterologous genes downstream from the phaseolin promoter. The phaseolin promoter has been used successfully to express the Brazil nut protein in soybeans, canola and tobacco.

A DNA sequence coding for S-antigen can be obtained using standard methods as described in Maniatis et al., op. cit. Alternatively a DNA sequence encoding S-antigen or interphotoreceptor retinoid-binding protein can be obtained by PCR amplification of S-antigen cDNA using specific primers. Briefly, cDNA synthesis of mRNA can be conducted using reverse transcriptase and specific primers. Primers can be designed from a known DNA sequence for S-antigen (GenBank Accession

X12453) or interphotoreceptor retinoid-binding protein (GenBank Accession M22453). Double stranded cDNA synthesis can be performed and adaptors can be ligated onto the ends of the cDNA sequence to provide for ease of cloning into a vector or cDNA
5 can be amplified using PCR and specific primers which incorporate restriction enzyme sites at their 5' ends to facilitated site directed cloning of the PCR product encoding the antigen into the compatibly cleaved vector. The cDNA sequences can then be introduced into a vector such as puc19 and
10 amplified in bacterial host cells. Transformants containing S-antigen or interphotoreceptor retinoid-binding protein inserts can be screened by hybridization to a probe designed based on a known DNA sequence for S-antigen or interphotoreceptor retinoid-binding protein.

15 Once the DNA sequence encoding the desired protein is isolated, the DNA sequence can be ligated into plant expression vectors at an appropriate cloning sites so that its expression is controlled by the phaseolin promoter. Plasmid, including a DNA sequence encoding S-antigen or interphotoreceptor
20 retinoid-binding protein, can be selected, amplified, and isolated by examining the restriction digestion patterns of plasmids from cells growing in kanamycin.

The DNA sequence coding for interphotoreceptor retinoid-binding protein can be obtained in another manner.
25 Briefly, mRNA from host tissue is isolated, poly-A selected and reverse transcribed with oligo dT priming. Single stranded cDNAs are tailed at 3' ends with oligo d(C) and primed with oligo d(G) and transcribed with reverse transcriptase or cDNA can be amplified using PCR and specific primers which
30 incorporate restriction enzyme sites at their 5' ends to facilitated site directed cloning of the PCR product encoding the antigen into the compatibility cleaved vector. Double stranded cDNAs are inserted at a restriction endonuclease site of a vector. The vectors are then transformed into a bacterial
35 host cell. Transformants having inserts encoding interphotoreceptor retinoid-binding protein can be identified by hybridization to probes designed from the known sequence of interphotoreceptor retinoid-binding protein. Once isolated and identified, cDNA sequence encoding interphotoreceptor

retinoid-binding protein can be subcloned from a plasmid such as puc19 to a binary vector.

Once obtained in a vector such as puc19, the DNA sequence coding for interphotoreceptor retinoid-binding protein can be subcloned in to a plant expression vector appropriate cloning site so that its expression is controlled by the phaseolin promoter. Alternatively, it can be subcloned immediately downstream from the DNA sequence coding for S-antigen to form a dicistronic construct under control of a single phaseolin promoter. Plasmid pPHI4752, including a DNA sequence encoding S-antigen, can be selected, amplified, and isolated as above.

The expression cassette can then be subcloned into a binary vector. The binary vector carrying the expression cassette coding for S-antigen and/or interphotoreceptor retinoid-binding protein is introduced in *Agrobacterium tumefaciens* LBA4404 (available from Clone Tech, Palo Alto, CA) or other disarmed *A. tumefaciens* strains by the freeze-thaw method.

Example 6

Agrobacterium Strains Having a Binary Vector Including a DNA Sequence Encoding S- Antigen and Interphotoreceptor Retinoid-binding Protein in Transgenic Soybean Plants

Transgenic soybean plants can be formed according to the method described in U.S. Patent Application Serial No. 07/920,409, the entire contents of which are hereby incorporated by reference. Soybean (*glycine max*) seed, is surface sterilized by exposure to chlorine gas evolved in a glass bell jar. The gas is produced by adding 3.5 mL hydrochloric acid (34 to 37% w/w) to 100 mL 5.25% w/w sodium hypochlorite. Exposure is for 16 to 20 hours in a container approximately one cubic foot in volume. Surface sterilized seed is stored in petri dishes at room temperature. Seed is germinated by plating 1/10 strength agar solidified medium according to Gambourg [B5 basal medium with minimal organics, Sigma Chemical Catalog No. G5893, 0.32 gm/L sucrose; 0.2% w/v and 3.0 mM 2-N-morpholino)ethanesulfonic acid (MES)] without plant growth regulators and culturing at 28°C with a 26-hour day length and cool white fluorescent illumination of approximately 20 $\mu\text{EM}^2 \text{S}^{-1}$. After three or four days, seed is prepared for co-cultivation. The seed coat is

removed and the elongating radicle is removed 3 to 4 mm below the cotyledons.

Overnight cultures of *Agrobacterium tumefaciens* strain LB4404 harboring the modified binary plasmid pPHI1680 as described above are grown to log phase in minimal A medium containing 1 microgram/mL tetracycline, are pooled, and an optical density measurement is taken at 550 nanometers. Sufficient volume of the culture is placed into 15 mm conical centrifuge tubes such that upon sedimentation between 1 and 2 $\times 10^{10}$ cells were collected in each tube where $DD=55=1.4 \times 10^9$ cells/mL. Sedimentation is by centrifugation at 6000 x g for ten minutes. After centrifugation, the supernatant is decanted and the tubes are held at room temperature until inoculum is need, but not longer than one hour.

Inoculations are conducted in batches such that each plate of seed is treated with a newly resuspended pellet of *Agrobacterium*. One at a time, the pellets are resuspended in 20 mL inoculation medium. The inoculation medium consists of 3.2 g/L B5 salts (G5893); 2.0% w/v sucrose; 45 μ moles 6-benzylaminopurine (BAP); 0.5 μ M indolebutyric acid (IBA); 100 μ M acetosyringone; and was buffered to pH 5.5 with 10 mM MES. Resuspension is by vortexing. The inoculum is then poured into a petri dish containing a prepared seed and the cotyledonary nodes are macerated with a surgical blade. This is accomplished by dividing the seed in half by the longitudinal section through the shoot apex, preserving the two whole cotyledons. The two halves of the shoot apex are then broken off of their respective cotyledons by prying them away with a surgical blade. The cotyledonary node is then macerated with a surgical blade by repeated scoring along the axis of symmetry. Care was taken not to cut entirely through the explant to the abaxial side. Explants are prepared in roughly about five minutes and then incubated for 30 minutes at room temperature without agitation. After 30 minutes, the explants are transferred into plates of the same medium solidified with 0.2% w/v/ Gelrite (Merck & Company, Inc.). Explants are imbedded with the adaxial side up and leveled with the surface of the medium and cultured at 22°C for three days under cool white fluorescent light, approximately 20 μ EM² S¹.

After three days, the explants are moved to liquid counterselection medium. The counterselection medium consisted of 3.2 g/L B5 salts, 2% w/v sucrose; 5 μ M BAP; 0.5 μ M IBA, 200 μ g/L vancomycin; 500 μ g/mL cefotaxime; and was buffered to pH 5.7 with 3 mM MES. Explants are washed in each petri dish with constant slow gyratory agitation at room temperature for four days. Counterselection medium is replaced four times.

The explants are then picked to agarose/solidified selection medium. The selection medium consisted of 3.2 g/L B5 salts, 2% w/v sucrose, 5.0 μ M BAP, 0.5 μ M IBA, 50 μ g/mL kanamycin sulfate, 100 μ g/mL vancomycin, 30 μ g/mL cefotaxime, 30 μ g/mL timentin, and is buffered to pH 5.7 with 3 mM MES. Selection medium was solidified with 0.3 w/v Seakem Agarose. The explants are imbedded in the medium, adaxial side down, and cultured at 28°C with a 16 hour day length in cool white fluorescent illumination of 60 to 80 μ EM² S⁻¹.

After two weeks the explants are again washed with liquid medium on the gyratory shaker. The wash is conducted overnight, in counterselection medium containing 50 μ g/mL kanamycin sulfate. The following day, explants are placed to agarose/solidified selection medium. They are imbedded in the medium with the adaxial side down and cultured for another two week period.

After one month on the on selected medium, transformed tissue is visible as green sectors of regenerating tissue against a background of bleached healthy tissue. Explants without green sectors are discarded, explants with green sectors are transferred to elongation medium. Elongation medium consists of 3.2 g/L B5 salts, 2% w/v sucrose, 3.3 μ M IBA, 1.7 μ M gibberellic acid, 100 μ g/mL vancomycin, 30 μ g/mL cefotaxime, and 30 μ g/mL timentin, buffered to pH 5.7 with 3 mM MES. Elongation medium is solidified with 0.2% w/v Gelrite. The green sectors are embedded with adaxial side up and cultured as before. Culture is continues on this medium with transfers to fresh plates every two weeks. When shoots are 0.5 cm in length they are excised at the base and placed in rooting medium in 13 x 100 ml test tubes. Rooting medium consists of 3.2 g/L B5 salts, 15 g/L sucrose, 20 μ M nicotinic acid, 900 mg/L pyroglutamic acid, and 10 μ M IBA. The rooting medium is buffered to pH 5.7 with

3 mM MES and solidified with 0.2% w/v Gelrite. After ten days, the shoots are transferred to the same medium without IBA or PGA. Shoots are rooted and held in these tubes under the same environmental conditions as before.

5 When a root system is well established, the plantlet is transferred to sterile soil mixed in plantcons. Temperature, photoperiod and light intensity remain the same as before.

The expression of S-antigen and/or interphotoreceptor retinoid-binding protein in transgenic soybean plants can be confirmed by PCR and quantitated using ELISA or Western blot analysis. Stability of expression can be evaluated by these same methods over successive generations.

Example 7

Formation of an Expression Cassette and Transgenic Sunflower Plant and Seeds Including the S-antigen and/or Interphotoreceptor Retinoid-binding Protein

15 An expression cassette encoding S-antigen and/or interphotoreceptor retinoid binding protein can be used to generate transgenic sunflower seeds and plants. The DNA sequence coding for S-antigen and/or interphotoreceptor retinoid-binding protein can be inserted into an expression cassette under control of the napin promoter for seeds specific expression.

25 Plasmid includes a plant transcription unit for the gene NPTII, which can be used in selecting transformed cells and appropriate cloning sites that provides for seed specific expression under control of the napin promoter. This promoter has been used successfully to express WGA and beta-glucuronidase genes in canola seeds.

30 A DNA sequence encoding S-antigen and/or interphotoreceptor retinoid-binding protein can be obtained as described in Example 5. The DNA sequence can be subcloned into the plant expression vector. Plasmids having a DNA sequence encoding S-antigen and/or interphotoreceptor retinoid binding protein be selected, amplified, and isolated by using phage cDNA libraries as described in Maniatis et al., A Guide to Molecular Cloning, op. cit. This expression cassette is then subcloned into a binary vector using the appropriate cloning sites and transferred to

Agrobacterium tumefaciens strain LBA4404.

Sunflower plants can be transformed with *Agrobacterium* strain LBA4404 by microparticle bombardment as described by Bidney et al., Plant Mol. Bio. 18:301 (1992). Briefly, sunflower seeds
5 are dehulled and the surface sterilized. The seeds are imbibed in the dark at 26°C for eighteen hours on filter paper moistened with water. The cotyledons and root radical are removed and meristem explants cultured on 374BGA medium (MS salts, Shephard vitamins, 40 ml/L adenine sulfate, 3% sucrose, 0.8% phytagar pH
10 5.6, plus 0.5 mg/L BAP, 0.25 ml/L IAA and 0.1 mg/L GA.) Twenty four hours later, the primary leaves are removed to expose the apical meristem and the explants are placed with the apical dome facing upward in a 2 cm circle in the circle of a 60 mm by 20 mm petri plate containing water agar. The explants are
15 bombarded twice with tungsten particles suspended in TE buffer as described above, or with particles associated with the plasmid. Some of the TE/particle bombardment explants are further treated with *Agrobacterium tumefaciens* strain carrying the plasmid by placing a droplet of bacteria suspended in the
20 inoculation medium, OD600 2.00, directly onto the meristem. The meristem explants are co-cultured on 374BGA medium in the light at 26°C for an additional 72 hours.

Agrobacterium treated meristems are transferred following the 72 hour co-culture period to medium 374 (374BGA with 1% sucrose
25 plus 50 mg/L kanamycin sulfate and no BAP, IAA or GA3) and supplemented with 250 µg/mL cefotaxime. The plantlets are allowed to develop for an additional two weeks under sixteen hour day and 26°C incubation conditions. Green or unbleached plantlets are transferred to medium 374 and grown until they
30 develop seed. The presence of S-antigen and interphotoreceptor retinoid-binding protein can be confirmed and quantitated as described in Example 5.

Animal Models for Autoimmune diseases

Currently, there are a number of animal models used for the
35 study of autoimmune diseases. Below is a list of some of these models.

Disease	Animal Models	Protein Fed
Multiple sclerosis	EAE in mouse or Lewis	MBP; PLP Type

- rats
- Rheumatoid arthritis Adjuvant Arthritis II collagen
in the rat. Collagen-
Induce Arthritis in mouse
or rats.
Transgenic mice chimeric for
human/mouse HLA-DR1.
- Uveoretinitis EAU in the mouse or Rat S-antigen; IRBP
- Type I diabetes Spontaneous IDDM in BB Insulin; GAD
Transgenic NOD mouse
- Myasthenia gravis EAMG in the Mouse AChR
- Thyroiditis EAT in the Mouse Thyroglobulin
- Transplantation Alloantigen;
MHC peptide
- Detailed protocols with a description of critical parameters and trouble shooting for the majority of these animal models are published in the Current Protocols in Immunology /ed. J. Coligan John Wiley & Sons, Inc. chapter 15 the entire contents of which are hereby incorporated by reference.
- Feeding Transgenic Plant Material to Mice with Relapsing-Remitting Experimental Autoimmune Encephalitis
- Experimental autoimmune encephalitis (EAE) is induced in six week old female SJL/J mice by inoculation of either MBP-protein or mouse spinal cord homogenate (MSCH), together with complete Freund's adjuvant. A second immunization is given one week later (53). The mice develop EAE in about 14-21 days and the first relapse is generally between 55 and 60 days. Animals showing clinical signs are graded and separated from asymptomatic animals. Animals with R-EAE (relapsing-remitting experimental autoimmune encephalitis) are divided into two groups, those that are fed transgenic plant material expressing MBP and those that are not. The controls include animals receiving complete Freund's adjuvant but not MBP or MSCH, and normal age and sex

match animals.

Mice are observed for clinical signs several days a week until the first relapse, at which time they are observed daily. The grading system used has been described by Kolowski et al. (54) and Kennedy et al. (55) in chronic EAE in SJL/J: Grade 0, no abnormality Grade 1, slow, sluggish; Grade 2, limp tail; Grade 3, limp tail and hind limb weakness (waddling gait); Grade 4, partial hind limb paralysis; Grade 5, complete hind limb paralysis; Grade 6, animal immobile; Grade 7, moribund. The length of time to relapse is evaluated and the change in grade, if present at the end of relapse, is assessed.

Two groups of eight R-EAE mice, one induced with MBP, the other with MSCH, receive different doses of either low dose (0.5 mg MBP) or high dose (20 mg MBP) plant expressed MBP over a one week period on day 21. Controls include R-EAE animals and normal animals. All animals are subjected to clinical and histological examination. No disease is induced in normal control animals treated with MBP.

This animal model displays many of the histopathological and clinical similarities to relapsing-remitting forms of MS (28). The mechanisms involved in the disease process have been the subject of intense study for several years using a mouse model of experimental autoimmune encephalitis (EAE).

EAE can be induced in SJL mice by injection of mouse spinal cord homogenate (MSCH), MBP, or PLP, the injection of synthetic peptides whose sequence corresponds to the major encephalitogenic epitopes of myelin basic protein MBP 84-104 and proteolipid protein PLP 139-151 or by adoptive transfer of activated CD4+ T_H1 cells, but not T_H2 cells, specific for encephalitogenic epitopes. A list of encephalitogenic peptides of MBP, PLP, and MOG are listed in table 15.1.1 of chapter 15 of Current Protocols in Immunology, supplement 19 (73). The disease is characterized by a relapsing-remitting course of neurological dysfunction (R-EAE), perivascular mononuclear infiltration and demyelination. CNS damage is mediated by inflammatory cytokines which can activate additional monocytes and macrophages non-specifically (29,30).

Although the initial attack in EAE can be induced by the administration of either T cells specific for MBP or for PLP,

close examination of reactivities of the T cells in the primary and subsequent relapses demonstrates the presence of T cells which interact with specificities other than the inducing epitopes. This expansion of encephalogenic epitopes is termed
5 determinant-spreading (30,31,32). A corollary of this observation is that antigen-specific treatment would be expected to be more effective when administered early in the course of disease, before the onset of increasing epitope complexity and eventual non-specific inflammation. The chronic or R-EAE is
10 best induced in SJL/J (H-2^u) or Lewis rats. The PL/J or H-2^u mouse is unlikely to have a relapsing course although EAE is more specifically a MBP induced disease in this strain than it is in SJL/J. The (SJL/J x PL/J) F1 also develops a chronic relapsing disease that has a combination of encephalogenic
15 epitopes that it sees (33, 34).

Clinical and Histological Status

In one experiment, animals are observed daily over a six month period in order to determine their clinical status, assessed as described above. Differences among experimental
20 groups are analyzed for statistical significance, using the Wilcoxon test for multiple comparisons of non-parametric case (clinical and histological scores) or paired Student's t test for mean time of relapse, mean length of relapse and mean clinical score at peak of relapse and remission. Correlations
25 between clinical and histological scores are evaluated using chi-square contingency analysis with Yates corrections as needed.

Of particular importance is complete prevention of relapses by MBP in animals in which EAE has been induced by MSCH as well
30 as MBP. MBP is efficacious for the animals in which disease was induced by MBP since determinant spreading in the MSCH-induced disease appears to focus mainly on secondary PLP epitopes.

In another experiment, animals, graded as to clinical condition, are sacrificed after 15, 30 and 90 days. The brain
35 and spinal cord are rapidly removed. A small area of each brain and two 5 mm areas of the spinal cord, cervical and thoracic, are fixed for histology and the degree of severity of the inflammatory response is graded by counting inflammatory foci.

Sections are taken from the same region of the brain and the cervical and thoracic of the spinal cord for each mouse. Grading of the inflammatory response is accomplished in 20 consequence fields and assessed 150 microns apart at 100 x magnification.

5 The response are graded as 1+ mild, 1-3 small foci; 2+, moderate, more than 3-7 foci and containing at least 10 cells per foci; 3+, severe, large foci of 15 to 25 cells including perivascular and meningeal collections. The studies are read in a blind fashion. Evaluation for histological scores include
10 mean grading of 20 sections per animal, and comparisons of individual animals by Student's t test. Clinical pathologic correlations are made comparing the clinical grade with the pathological score after the code is broken. Statistical analysis has been described above.

15 Quantitation of Dose

In order to establish the minimum dose of antigen required to affect the disease process and determine the threshold below which the MBP protein is ineffective or antigenic, a variety of doses are administered. Both high and low-expressing MPB plants
20 are isolated and the exact amount of MBP protein produced is assayed using ELISA with a primary MBP-specific antibody and MBP material expressed in the baculovirus system. Then, low dose and high dose expressing plants are fed to R-EAE animals whose disease was induced with MBP. Since there is no difference
25 between these two experimental sets of animals, the dose of the low expressions is decreased until the efficacy of treatment has been significantly diminished or altered. Having ascertained the minimum effective dose, this quantity of protein is then delivered to animals to determine if the course of the disease
30 is different. Blood samples are assayed by ELISA to determine the amount of MBP protein detectable in serum. Although a single seven day treatment generally produces permanent inhibition of subsequent relapses, the MBP can be administered daily until the controls relapse.

35 Prevention of EAE

Experiments are conducted to determine if EAE can be reintroduced in treated animals and if feeding plant expressed

MBP prevents the disease from spreading to encephalogenic epitopes of proteolipid protein (PLP). PLP is another important myelin protein implicated in MS and known to produce EAE in mice.

5 Ablation of MBP Expression

To study efficacy of MBP protein as a therapy for MS, SJL mice are treated with antibody to MBP. The alteration of serum concentration of MBP is measured and compared with the clinical and histological status of the mice. If the mice suffer relapse, extinction of the protein becomes a critical parameter and serum MBP is important. If the mice do not relapse, they are again challenged with either MBP or MSCH in order to determine whether disease can be reinduced and whether the severity of the disease has been altered.

15 The protocol of Lindsey et al. (56) is followed with the highest reinduction at 12 weeks. R-EAE mice induced by MBP or MSCH are used and reinduced just prior to the second relapse.

Although the type of plant into which the cDNA for MBP is introduced is not critical, potatoes have been found to be particularly useful for generating this antigen. Potato contains a large percentage of protein/gram weight of tissue. There already exists a tuber specific promoter from a gene encoding patatin; this family of protein accounts for 40% of the protein in potato tubers (52). Expression with this promoter directs synthesis of the MBP in the tuber portion of the potato. Additional benefits of using potato include the short amount of time required to generate transgenic plants and tube material (approximately three months for micro-tuber material). Although a more palatable delivery vehicle may be in the form of vegetable or tomato juice or an edible fruit or vegetable, the potato material can be incorporated into a shake and flavored according to personal preference by addition of strawberries, bananas, etc. Additional plant promoters and plant expression vectors have been reported in the literature that can also be used. Researchers have succeeded in producing recombinant proteins in plant seeds that constitute over ten percent of the seed's total protein. In soybeans this accounts for four percent of the dry weight of the seed. Foreign proteins

expressed in this system are protected from degradation due to the natural environment of the seed which is lyophilized and low in proteases.

Preferred plant promoters are those that express the foreign protein at about 0.1 to about 10% of the total plant protein. Based upon nutrient analysis figures obtained from the USDA, a potato weighing 202 grams is comprised of 71% water, 25% carbohydrate, 2.5% protein, with the remaining portion being comprised of various nutrients. Assuming expression levels between 10 and 0.1% of the total protein, this results in 500 mg to 5 mg per MBP potato, respectively. The current dose used in human clinical trials for treatment of MS is 300 mg of bovine derived myelin containing approximately 7.5 mg of MBP and 15 mg of PLP(6, 57, 60) or a dose of 0.1 to 0.5 mg of chicken type II collagen or 1 to 10 mg of bovine type II collagen for the treatment of rheumatoid arthritis (59). Recent studies on the effects of low dose (ranging from 0.5 mg to 5 mg) vs. high dose (ranging from 20 mg to 500 mg) induction of tolerance in rats or mice indicate active suppression at low doses and anergy and/or deletion at high doses (2,3,4). As demonstrated in animal models, administration at high enough doses causes apoptosis, presumably caused by high concentration of IL2 and antigen (2,4). Finally, the delivery of the antigen by consumption of the transgenic plant may facilitate its passing through the stomach and into the gut and thereby enhance the presentation and uptake in a soluble form that would induce tolerance.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various application such specific embodiments without departing from the generic concept, and therefore such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation.

All references cited in this specification are hereby incorporated by reference.

LITERATURE CITED

1. Wells, H.G. 1911. *J. Infect. Dis.* 8:147-171.
2. Critchfield, J.M., M.K. Racke, J.C. Zúñiga-Pflücker, B. Cannella, C.S. Raine, J. Goverman and M.J. Lenardo 1993. *Science*. 263:1139-1143.
3. Chen, Y.H, J. Inobe, V.K. Kuchroo, J.L. Baron, C.A. Janeway and H.L. Weiner. 1996. *PNAS* 93:388-391.
4. Gienapp, I., K. Cox, N. Javed and C. Whitacre. 1996. *Ann.N.Y. Acad. Sci.* 778:382-383.
5. Weiner, H.L., A. Friedman, A. Miller, S.J. Khoury, A. Alsabbagh, L. Santos, M. Sayegh, R.B. Nussenblatt, D.E. Trentham and D. A. Hafler 1994. *Annu. Rev. Immunol.* 12:809-837.
6. Hoyne, G.F. and J.R. Lamb. 1996. *Immunol & Cell Biol.* 74:180-186.
7. Weiner, H.L., G.A. Mackin, M. Matsui, E.J. Orav, S.J. Khoury, D.M. Dawson and D.A. Hafler. 1993. *Science*. 259:1321-1324.
8. Sieper, J., S. Kary, H. Sorensen, R. Alten, U. Eggens, W. Hüge, F. Hiepe, A. Kuhne, J. Listing, et al. 1996. *Arthritis and Rheumatism*. 39:41-51.
9. Bernard, C.C.A., and N.K. de Rosbo. 1992. *Curr. Opin. Immunol.* 4:760-765.
10. Frech-Constant, C. 1994. *Lancet* 343:271-275.
11. Martin, R., H.F. McFarland and D.E. McFarlin. 1992. *Annu. Rev. Immunol.* 10:153-187.
12. Wucherpfenning, K.W., H.L. Weiner and D.A. Hafler. 1991. *Immunol. Today* 12:277-282.
13. LaSalle, J.M., K. Ota and D.A. Hafler. 1991. *Immunol.* 147:774-780.
14. LaSalle, J.A., P.J. Tolentinao, G.J. Freeman, L.M. Nadler and D.A. Hafler. 1992. *J.Exp.Med.* 176:177-186.
15. Correale, J., M. McMillan, K. McCarthy, T. Le and L.P. Weiner. 1995. *Neurology* 45:1370-1378.
16. Olsson, T. 1992. *Curr. Opin. Neurol. & Neurosurgery* 5:195-202.
17. Raine, C.S. 1994. *Ann. Neurol.* 36:S61-S72.
18. Raine, C.S. 1991. *Neuropathol. Appl. Neurobiol.* 17:265-274.
19. Mosmann, T.R. and R.L. Coffman. 1989. *Annu. Rev.*

Immunol. 7:145-173.

20. Germain, R.N. 1994. Cell. 76:287-299.

21. Mueller, D.L., M.K. Jenkins and R.H. Schwartz. 1989. Annu. Rev. Immunol. 7:445-480.

5 22. Miller, S.D., B.L. McRae, C.L. Vanderlugt, K.M. Nikceovich, J.G. Pope, L. Pope and W.J. Karpus. 1995. Immunol. Rev. 144:225-244.

23. McRae, B.L., C.L. Vanderlugt, M.C. Dal Canto and S.D. Miller. 1995. J. Exp. Med. 182:75-85.

10 24. Sprent, E.K., Gao and S.R. Webb. 1990. Science. 248:1357-1361.

25. Gorman, J., A. Woods, L. Larson, L.P. Weiner, L.E. Hood and D.M. Zaller. 1993. Cell. 72:551-560.

15 26. Lafaille, J.J., K. Nagashima, M. Katusuki and S. Tonegawa. 1994. Cell. 78:399-408.

27. Hickey, W.F. 1991. Brain Pathol. 1:97-105.

28. Zamvil, S.S. and L. Steinman. 1990. Annu. Rev. Immunol. 8:579-621.

29. Blalock, J.E. 1994. The Immunologist 2:8-15.

20 30. Miller, S.D. and W.J. Karpus. 1994. Immunol. Today 15:356-361.

31. Lehmann, P.V., T. Forsthuber, A. Miller and E.E. Sercarz. 1992. Nature. 358:155-157.

25 32. Jiang, H., S-I Zhang and B. Pernis. 1992. Science. 256:1213-1215.

33. Whitham, R.H., B.L. Kotzin, A.C. Buenafe, A.D. Weinberg, R.E. Jones, G.A. Hashim, C.M. Hoy, A.A. Vandenbark and H. Offner. 1993. J. Neurosci. Res. 35:115-128.

30 34. Fritz, R.B., C-H. Jen Chou and D.E. McFarlin. 1983. J. Immunol. 130:191-194.

35. Franco, A., G.Y. Ishioka, L. Adorini, J. Alexander, J. Ruppert, K. Snoke, H.M. Grey and A. Sette. 1994. The Immunologist. 2:97-102.

36. Ishioka, G.Y., L. Adorini, J-C. Guery, F.C.A. Gaeta, R. LaFond, J. Alexander, M.F. Powell, A. Sette and H.M. Grey. 1994. J. Immunol. 152:4310-4319.

37. Zhao, W., K.W. Wegmann, J.L. Trotter, K. Ueno and W.F. Hickey. 1994. J. Immunol. 153:901-909.

38. Jansson, L., P. Diener, A. Engstrom, T. Olsson and R.

- Holmadahl. 1995. Eur. J. Immunol. 25:2195-2200.
39. Jameson, S.C., F.R. Carbone and M.J. Bevan. 1993. J. Exp. Med. 177:1541-1550.
40. Karin, N., D.J. Mitchell, S. Brocke, N. Ling and L. Steinman. 1994. J. Exp. Med. 180:2227-2237.
41. Kuchroo, V.K., J.M. Greer, D. Kaul, G. Ishioka, A. Franco, A. Sette, R.A. Sobel and M.B. Lees. 1994. J. Immunol. 153:3326-3336.
42. Gaur, A., B. Wiers, A. Lui, J. Rothbard and C.G. Fathman. 1992. Science 258:1491-1494.
43. Racke, M.K., J.M. Critchfield, L. Quigley, B. Cannella, C.S. Raine, H.F. McFarland and M.J. Lenardo. 1996. Ann. Neurol. 39:46-56.
44. Metzler, B. and D.C. Wraith. 1993. Int. Immunol. 5:1159-1165.
45. Khoury, S.J., M.H. Sayegh, W.W. Hancock, L. Gallon, C.B. Carpenter and H.L. Weiner. 1993. J. Exp. Med. 178:559-566.
46. Gaur, A., B. Wiers, A. Lui, J. Rothbard and C.G. Fathman. 1992. Science 258:1492-1494.
47. Mitchison, N.A. 1968. Immunol. 15:509-516.
48. Chen, Y., J.I. Inobe, R. Marks, P. Gonnella, V.K. Kuchroo and H.L. Weiner. 1995. Nature 376:177-180.
49. Tariq, A.H., H.S. Mason, J.D. Clements and C.J. Arntzen. 1995. Science 268:714-716.
50. Mason, H.S., J.M. Ball, J-J Shi, M.K. Estes and C.J. Arntzen. 1996. PNAS 93:1-16.
51. Wenzler, H., G. Mignery, G. May and W. Park. 1989. Plant Sci. 63:79-85.
52. Mignery, G.A., C.S. Pikaard, D.J. Hannapel and W.D. Park. 1984. NAR 12:7987-8000.
53. Brown, A.M. and D.E. McFarlin. 1981. Laboratory Invest. 45:278-284.
54. Kozlowowski, P.B., G.B. Schuller-Levisn and H.M. Wisniewski. 1987. Acta Neuropathologica 74:163-168.
55. Kennedy, M.K., L.J. Tan, M.C. Dal Canto, V.K. Tuohy, Z. Lu, J.L. Trotter and S.D. Miller. 1990. J. Immunol. 144:909-919.
56. Lindsey, J.W., M. Pappolla and L. Steinman. 1995. Cell Immunol. 162:235-240.
57. Hohol, M.J., S.J. Khoury, S.L. Cook, E.J. Orav, D.A.

- Hafler and H.L. Weiner. 1996 Ann.N.Y.Acad.Sci. 778:243-250.
58. Trentham, D.E., R.A. Dynesius-Trentham, E.J. Orav, D. Combitchi, C. Lorenzo, K.L. Sewell, D.A. Hafler and H.L. Weiner 1993. Science 261:1727-1729.
- 5 59. Miller, A., O. Lider, A. Al-Sabbagh and H.L. Weiner. 1992. J. Neuroimmunology, 39:242-250.
60. Kent, S.C., H. Fukaura, M.J. Pietrusewicz, S.J. Khoury, H.L. Weiner and D.A. Hafler 1997. Ann.N.Y.AcadSci. Apr5;815:412-422.
- 10 61. Sergio, A.J., L. Ala-Kokko, D.J. Prockop, C.F. Merryman, N. Shepard and G. R. Dodge 1997. Matrix Biology 16:29-39.
62. Baldwin, C.T., A. m. Reginato, C. Smith, S.A. Jimenez and D.J. Prockop 1989. Biochem.J. 262:521-528.
63. Hassler, S. 1997. Nature Biotechnology 15:597.
- 15 64. Goodman, R.M., V.C. Knauf, C.M. Houck and L. Comai 1990. US pat. 4,956,282.
65. Vendkerchone 1989
66. Sijmons, P.C., B.M. Dekker, B. Schrammeijer T.C. Verwoerd, P.J. van den Elzen and A Hoekema 1990. Biotechnology 8(3):217-221.
- 20 67. Hiatt 1990. Nature 344(6265):469-470
68. Mason, H.S., D.M.K. Lam and C.J. Arntzen 1995. PNAS89:11745-11749.
69. Ma, S.W., D.L. Zhao, Z.Q. Yin, R. Mukherjee, B. Singh, H.Y. Qin, C.R. Stiller and A.M. Jevnikar. 1997. Nat.Med. Jul;3(7):793-796.
- 25 70. Warren, K.G. and I. Catz 1997. J.Neurol Sci May1;148(1):67-78.
71. Rosloniec, E.F., D.D. Brand, L.K. Myers, K.B. Whittington, M. Gumanovskaya, D.M. Zaller, A. Woods, D.M. Altmann, J.M. Stuart and A.H. Kang. 1997 J.Exp.Med. 185:1113-1122
- 30 72. Clinical trials update Genetic Engineering News May 15 1997, Vol17.No. 10 p.36.
73. Current Protocols in Immunology 1997 John Wiley & Sons, Inc.
- 35 74. Weiner, H.L. and L.F. Mayer. 1996 Annals NY.Acad.Sci. Vol.778.

WHAT IS CLAIMED IS:

1. A method for preventing, inhibiting or treating an immune-mediated condition selected from the group consisting of autoimmune diseases, allergic reactions and transplant rejections, comprising feeding to an animal vulnerable to or suffering from said disorder, in the form of a food an effective amount of a tolerogenic antigen specific to said condition and produced by at least one transgenic plant, said food being said plant or a food product derived from said plant.
2. The method of claim 1 wherein the tolerogenic antigen is an autoimmune antigen and an autoimmune disease is prevented or treated.
3. The method according to claim 2 wherein the autoimmune antigen is selected from the group consisting of human myelin basic protein, type II collagen, myelin proteolipid protein, S-antigen, interphotoreceptor binding protein, insulin, glutamate decarboxylase, acetylcholine receptor, and thyroglobulin.
4. The method of claim 1, wherein the tolerogenic antigen is an allergen and an allergic reaction is prevented or treated.
5. The method according to claim 8 wherein the tolerogenic antigen is selected from the group of allergens consisting of bee venom peptide PLA-2, feline antigen Feld1, MHC alloantigen, and 70-80 kD mite allergen.
6. The method according to claim 1 wherein said transgenic plant is an edible plant.
7. The method according to claim 6 wherein the transgenic plant is selected from the group consisting of potatoes, corn, sunflower seeds, alfalfa, peas, peanuts, tomatoes, broccoli, spinach, bananas, beans, green bean, lima beans, carrots, and rice.
8. The method of claim 1 in which the animal is a human.
9. Transgenic plants expressing an antigen selected from the group consisting of autoimmune antigens, allergens, and mixtures thereof.
10. A transgenic plant according to claim 9 wherein the autoimmune antigen is selected from the group consisting of human myelin basic protein, type II collagen, myelin proteolipid protein, S-antigen, interphotoreceptor binding protein, insulin,

glutamate decarboxylase, acetylcholine receptor, and thyroglobulin, and the allergen is selected from the group consisting of bee venom peptide PLA-2, feline antigen Feld1, MHC alloantigen, and 70-80 kD mite allergen.

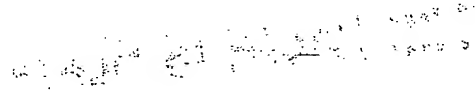
5 11. A transgenic plant according to claim 9 wherein the transgenic plant is an edible plant.

10 12. A transgenic plant according to claim 11 wherein the plant is selected from the group consisting of potatoes, corn, sunflower seeds, alfalfa, peas, peanuts, tomatoes, broccoli, spinach, bananas, beans, green beam, lima beans, carrots, and rice.

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(54) Title: DELIVERY OF TOLEROGENIC ANTIGENS VIA EDIBLE PLANTS OR PLANT-DERIVED PRODUCTS (57) Abstract Autoantigens and allergens can be expressed in transgenic plants, and the plants, or products derived therefrom, used as foods or beverages to prevent or treat autoimmune diseases or allergic reactions.		

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BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/13634

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C07K14/47 C07K14/435 C12Q1/68 A61K39/00
A01K67/027 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12Q A61K A01K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 08347 A (LONDON HEALTH ASS ;JEVNIKAR ANTHONY M (CA); MA SHENGWU (CA); STILL) 30 March 1995	1-4,6-12
Y	pages 1-2; page 3, line 1-8; page 4, lines 1-25; page 10, lines 15-25, Example 8; claim 7	1,4,6-12
Y	--- WO 92 06708 A (BRIGHAM & WOMENS HOSPITAL) 30 April 1992 especially page 2, line 15-31; page 3; page 7; claim 2 see the whole document	1,4,6-12
A	--- WO 95 30435 A (IMMULOGIC PHARMA CORP) 16 November 1995 see the whole document ---	1-3,6-12
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

9 March 1998

Date of mailing of the international search report

11.03.98

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/13634

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No. —
A	MASON H S ET AL: "TRANSGENIC PLANTS AS VACCINE PRODUCTION SYSTEMS" TRENDS IN BIOTECHNOLOGY, vol. 13, no. 9, September 1995, pages 388-392, XP002024035 see page 392, left-hand column ---	1-3,6-12
A	WO 95 27500 A (BRIGHAM & WOMENS HOSPITAL) 19 October 1995 see the whole document ---	1-3,6-12
A	WO 96 12737 A (IMMULOGIC PHARMA CORP ;SMILEK DAWN (US); SAMSON MICHAEL (US); GEFT) 2 May 1996 see the whole document ---	1-3,6-12
A	WO 94 20135 A (EDIBLE VACCINES INC ;LAM DOMINIC MAN KIT (US); ARNTZEN CHARLES JOE) 15 September 1994 see page 8, line 22 - line 32 ---	1-3,6-12
A	MUELLER U ET AL: "SUCCESFUL IMMUNOTHERAPY WITH T-CELL EPITOPES OF BEE VENOM PHOSPHOLIPASE A2 (PLA) IN PATIENTS WITH BEE VENOM (BV) ALLERGY" JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, vol. 97, no. 1, PART 03, January 1996, page 426 XP002035179 see the whole document ---	1,4-12
A	GRIFFITH I J ET AL: "EXPRESSION AND GENOMIC STRUCTURE OF THE GENES ENCODING FDI, THE MAJOR ALLERGEN FROM THE DOMESTIC CAT" GENE, vol. 113, 1992, pages 263-268, XP002033869 see the whole document ---	1,4-12
A	BRINER, T.J., ET AL.: "PERIPHERAL T-CELL TOLERANCE INDUCED IN NAIVE AND PRIMED MICE BY SUBCUTANEOUS INJECTION OF PEPTIDES FROM MAJOR CAT ALLERGEN FEL d1" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 90, August 1993, pages 7608-7612, XP002056822 see the whole document ---	1,4-12

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INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/US 97/13634

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAYEGH, M.H., ET AL.: "INDUCTION OF IMMUNITY AND ORAL TOLERANCE WITH POLYMORPHIC CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ALLOPEPTIDES IN THE RAT" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, August 1992, pages 7762-7766, XP002056823 ESPECIALLY PAGE 7765, RIGHT COLUMN see the whole document ---	1,4-12
A	WO 90 08161 A (WISCONSIN BLOOD CENTER) 26 July 1990 PAGES 2,4,5,6, ---	1,4-12
A	WO 95 28424 A (IMMULOGIC PHARMA CORP) 26 October 1995 see the whole document ---	1,4-12
P,X	WO 96 40229 A (UNIV JEFFERSON ;US OF AMERICA REPRESENTED BY I (US)) 19 December 1996 see page 4 ---	1
P,X	MA, S.-W., ET AL.: "TRANSGENIC PLANTS EXPRESSING AUTOANTIGENS FED TO MICE TO INDUCE ORAL IMMUNE TOLERANCE" NATURE MEDICINE, vol. 3, no. 7, July 1997, pages 793-796, XP002056824 see the whole document -----	1,2,6-9, 11,12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/13634

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 1-8 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
The method of claim 5 was read as referring to claim 4 and not claim 8.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
1-12; (inventions 1 and 7, see continuation-sheet)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3,6-12

Providing an alternative method for the treatment of an autoimmune disease by suppressing the immune response; utilizing an edible transgenic plant expressing an autoantigen related to Multiple Sclerosis (MS) and/or Experimental Autoimmune Encephalomyelitis (EAE) and feeding the plant or parts thereof to animals and/or humans which suffer from or are vulnerable to said disease.

2. Claims: 1-3,6-12

Providing an alternative method for the treatment of an autoimmune disease by suppressing the immune response; utilizing an edible transgenic plant expressing an autoantigen related to Rheumatoid Arthritis and feeding the plant or parts thereof to animals and/or humans which suffer from or are vulnerable to said disease.

3. Claims: 1-3,6-12

Providing an alternative method for the treatment of an autoimmune disease by suppressing the immune response; utilizing an edible transgenic plant expressing an autoantigen related to Uveoretinitis and feeding the plant or parts thereof to animals and/or humans which suffer from or are vulnerable to said disease.

4. Claims: 1-3,6-12

Providing an alternative method for the treatment of an autoimmune disease by suppressing the immune response; utilizing an edible transgenic plant expressing an autoantigen related to Type I Diabetes and feeding the plant or parts thereof to animals and/or humans which suffer from or are vulnerable to said disease.

5. Claims: 1-3,6-12

Providing an alternative method for the treatment of an autoimmune disease by suppressing the immune response; utilizing an edible transgenic plant expressing an autoantigen related to Myasthenia and feeding the plant or parts thereof to animals and/or humans which suffer from or are vulnerable to said disease.

6. Claims: 1-3,6-12

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Providing an alternative method for the treatment of an autoimmune disease by suppressing the immune response; utilizing an edible transgenic plant expressing an autoantigen related to Thyroiditis and feeding the plant or parts thereof to animals and/or humans which suffer from or are vulnerable to said disease.

7. Claims: 1,4-12

Providing a method for the treatment of allergies by suppressing the immune response; utilizing an edible transgenic plant expressing an autoantigen related to allergies and feeding the plant or parts thereof to animals and/or humans which suffer from or are vulnerable to said disease.

8. Claims: 1,6,7,8

Providing a method for the treatment of transplant rejection by suppressing the immune response; utilizing an edible transgenic plant expressing an autoantigen related to transplant rejection and feeding the plant or parts thereof to animals and/or humans which suffer from or are vulnerable to said disease.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/13634

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9508347 A	30-03-1995	AU 7736794 A CA 2172398 A CN 1135718 A EP 0720484 A JP 9507743 T	10-04-1995 30-03-1995 13-11-1996 10-07-1996 12-08-1997
WO 9206708 A	30-04-1992	AU 3040995 A AU 9023791 A CA 2092905 A EP 0553291 A HU 69942 A IL 99754 A JP 2635444 B US 5720955 A US 5399347 A	30-11-1995 20-05-1992 16-04-1992 04-08-1993 28-09-1995 04-08-1996 30-07-1997 24-02-1998 21-03-1995
WO 9530435 A	16-11-1995	AU 2470595 A CZ 9603295 A EP 0758902 A HU 76099 A JP 10500109 T PL 317197 A SI 9520059 A SK 145896 A ZA 9503730 A AU 4278296 A CA 2203629 A CZ 9701226 A EP 0787147 A FI 971750 A NO 971900 A WO 9612737 A ZA 9509033 A	29-11-1995 14-05-1997 26-02-1997 30-06-1997 06-01-1998 17-03-1997 31-08-1997 07-05-1997 11-01-1996 15-05-1996 02-05-1996 17-09-1997 06-08-1997 24-06-1997 25-06-1997 02-05-1996 11-06-1996
WO 9527500 A	19-10-1995	AU 2246795 A AU 2277695 A BR 9507451 A BR 9507452 A CA 2185353 A CA 2187345 A	30-10-1995 30-10-1995 05-08-1997 05-08-1997 19-10-1995 19-10-1995

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/13634

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9527500 A		EP 0752879 A	15-01-1997
		EP 0752880 A	15-01-1997
		HU 74900 A	28-02-1997
		HU 76101 A	30-06-1997
		JP 9511745 T	25-11-1997
		JP 9511990 T	02-12-1997
		NO 964199 A	03-10-1996
		NO 964200 A	03-10-1996
		WO 9527499 A	19-10-1995
WO 9612737 A	02-05-1996	AU 2470595 A	29-11-1995
		AU 4278296 A	15-05-1996
		CA 2203629 A	02-05-1996
		CZ 9701226 A	17-09-1997
		CZ 9603295 A	14-05-1997
		EP 0758902 A	26-02-1997
		EP 0787147 A	06-08-1997
		FI 971750 A	24-06-1997
		HU 76099 A	30-06-1997
		JP 10500109 T	06-01-1998
		NO 971900 A	25-06-1997
		PL 317197 A	17-03-1997
		SI 9520059 A	31-08-1997
		SK 145896 A	07-05-1997
		WO 9530435 A	16-11-1995
		ZA 9509033 A	11-06-1996
WO 9420135 A	15-09-1994	US 5612487 A	18-03-1997
		AU 6813394 A	26-09-1994
		EP 0728014 A	28-08-1996
WO 9008161 A	26-07-1990	AU 4948890 A	13-08-1990
WO 9528424 A	26-10-1995	US 5611601 A	18-03-1997
		AU 2286295 A	10-11-1995
		EP 0755407 A	29-01-1997
		JP 9512014 T	02-12-1997
WO 9640229 A	19-12-1996	NONE	